

Tetronic® 904-containing polymeric micelles overcome the overexpression of ABCG2 in the blood–brain barrier of rats and boost the penetration of the antiretroviral efavirenz into the CNS

Aim: To assess the involvement of ABCG2 in the pharmacokinetics of efavirenz in the blood–brain barrier (BBB) and investigate a nanotechnology strategy to overcome its overexpression under a model of chronic oral administration. **Materials & methods:** A model of chronic efavirenz (EFV) administration was established in male Sprague–Dawley rats treated with a daily oral dose over 5 days. Then, different treatments were conducted and drug concentrations in plasma and brain measured. **Results:** Chronic treatment with oral EFV led to the overexpression of ABCG2 in the BBB that was reverted after a brief washout period. Moreover, gefitinib and the polymeric amphiphile Tetronic® 904 significantly inhibited the activity of the pump and potentiated the accumulation of EFV in CNS. The same effect was observed when the drug was administered within mixed micelles containing Tetronic T904 as the main component. **Conclusion:** Tetronic 904-containing polymeric micelles overcame the overexpression of ABCG2 in the BBB caused by chronic administration of EFV then boosting its penetration into the CNS.

Keywords: ABCG2 pump inhibition • CNS • drug-loaded poly(ethylene oxide)-b-poly(propylene oxide) polymeric micelles • efavirenz • HIV

According to the latest report on the global AIDS epidemic by WHO, more than 35 million people are living with the HIV [1]. The high activity antiretroviral therapy inhibits more effectively viral replication than monotherapy and not only prevents AIDS-related illness and death [2,3] but also significantly reduces the risk of HIV transmission between high-risk populations and the spreading of the epidemic [4]. However, suboptimal adherence, toxicity, drug resistance and viral reservoirs make the lifelong treatment of HIV infection challenging [5]. Moreover, even optimal treatment with high activity antiretroviral therapy does not lead to the eradication of the HIV from the host, owing to the generation of intracellular and anatomical reservoirs [6–8].

At present, although severe forms of HIV-associated neurocognitive disorders (e.g., HIV-associated dementia) have almost disappeared from the clinical practice, the

prevalence of other mild neurocognitive disorders (e.g., asymptomatic neurocognitive impairment) remains unchanged, suggesting that virus suppression in the brain is not effective [9]. These disorders have been associated with a lower survival rate and can greatly affect the cognitive development and the quality of life of individuals infected with HIV, especially children [10]. One potential explanation for the high prevalence of these disorders is the limited ability of several antiretroviral drugs to enter the brain. A ranking system for the effectiveness with which drugs penetrate into the CNS has demonstrated that patients generally exhibited a lower viral load in the cerebrospinal fluid while receiving antiretrovirals with greater brain-penetrating ability [8] and that this is translated into better neurocognitive outcomes [11]. These findings support the concept that the use of antiretroviral regimens with an improved ability to enter the brain could reduce viral loads

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in the brain and ultimately prevent HIV-associated neurological complications [12]. However, the presence of an intact blood–brain barrier (BBB) has long been known to restrict the penetration of antiretrovirals into the brain [13]. In addition to the presence of tight junctions, one mechanism by which the BBB precludes drug access into the CNS is the expression of different membrane-associated drug efflux transporters in brain microvessel endothelial cells [14]. In particular, the activity of transmembrane proteins of the ATP-binding cassette superfamily (ABCs) is intimately associated with the attainment of subtherapeutic antiretroviral concentrations in several HIV reservoirs [15,16]. Moreover, the overexpression of ABCs is a well-known mechanism of multidrug resistance in cancer and infectious diseases (e.g., viral hepatitis and HIV) [17].

Efavirenz (EFV) is a first-line antiretroviral prescribed to adults and children infected with HIV [18–20] but it demands therapeutic drug monitoring (TDM) due to relatively high inter- and intra-individual variability [20,21]. ABCG2 (also known as breast cancer resistant protein, BCRP) is highly expressed in various tissues. Previously, we have shown that the intestinal permeability of EFV is precluded by the activity of ABCG2 that pumps the drug in the basolateral-to-apical direction [22]. Furthermore, *in vitro* studies demonstrated that chronic treatment with EFV, among other antiretrovirals, could indirectly induce the expression of another ubiquitous ABC, namely ABCB1 (P-glycoprotein, P-gp) by activation of ligand-activated nuclear receptors known to regulate the expression of this efflux pump [12].

In this scenario, the investigation of ABC blockers has become a niche of intensive research to improve the bioavailability of drugs and overcome resistance, especially by using ‘inert’ amphiphilic pharmaceutical excipients [17,23,24].

Previous investigations demonstrated the high EFV nanoencapsulation capacity of polymeric micelles made of different poly(ethylene oxide)–*b*-poly(propylene oxide) block copolymers such as the linear Pluronic® F127 (F127) and the branched Tetronic® 904 (T904), with water solubility increases above 8000-fold [25–28]. EFV-loaded micelles were physically stable even under extreme dilution conditions [25,28] and led to a sharp increase of the oral bioavailability by 50–70% and reduction of the interindividual variability of the antiretroviral [25,26]. Intriguingly, EFV-loaded mixed polymeric micelles containing T904, a derivative that inhibits the activity of ABCG2 *in vitro* [27], boosted the plasma concentration of the drug with respect to counterpart micelles deprived of it [26]. Moreover, EFV-loaded micelles administered intranasally led to a significant increase of the drug bioavailability in the CNS

with respect to the intravenous route [29], stressing the great potential of this Trojan nanotechnology platform for encapsulation and release of poorly water-soluble anti-HIV drugs.

The goal of this work was to explore a simple and translatable nanotechnology strategy to overcome ABCG2 resistance in the BBB. Thus, we initially developed a model of EFV chronic oral administration that led to transient overexpression of this pump in the BBB and then comprehensively investigated the ability of EFV-loaded polymeric micelles with and without T904 to increase the CNS bioavailability of the drug upon iv. administration. Overall results demonstrate the ability of amphiphilic copolymers to overcome the overexpression of ABCs and to improve the pharmacokinetics of their substrates.

Materials & methods

Materials

F127 (12.6 kg/mol, 70% w/w PEO) and T904 (6.7 kg/mol, 40% w/w PEO) were donated by BASF (Florham Park, NJ, USA) and EFV by LKM Laboratories (Buenos Aires, Argentina). Gefitinib was isolated from Iressa® 250 mg coated tablets (Astra-Zeneca, Cheshire, UK). Complete mini (Roche, Applied Science, Mannheim, Germany) and phenylmethylsulfonyl fluoride (Sigma-Aldrich, MO, USA) and all the other chemicals not listed were of the highest purity available and were used as received.

Methods

Preparation of EFV-free & EFV-loaded polymeric micelles

Briefly, 10% w/v polymeric micelles were produced by dissolving the required amount of copolymer (1 g of F127 for single micelles or 0.25 g F127 + 0.75 g T904 for mixed micelles) in phosphate buffer solution (pH 7.4, 10 ml) at 4°C. Micelles were equilibrated at 25°C at least 24 h before encapsulation of the drug. To prepare EFV-loaded micelles (20 mg/ml, 2% w/v), 60 mg of drug was added to 10% single or mixed micelles (3 ml) and shaken (25°C) until complete drug dissolution (~2 h). The encapsulation capacity of these micelles was established in previous studies and the drug concentration in the formulations used in this work contained always 2% w/v that is below the saturation limit of the micelles [25–27]. Suspensions were filtered (0.45 µm, cellulose nitrate filters, Osmonics, Inc., Minneapolis, MN, USA) to remove any insoluble residue and stored at 23°C until use.

Single F127 and mixed F127/T904 polymeric micelles are named pF127-EFV and F127:T904-EFV, respectively. The size, size distribution and zeta potential of the EFV-loaded polymeric micelles were

characterized as reported elsewhere [25–27]. All the EFV-loaded micelles showed monomodal size distributions at 37°C with sizes of ~25 nm for pF127-EFV and 255 nm for F127:T904-EFV and polydispersity <0.4. The zeta potential was close to neutral due to the nonionic nature of the copolymers.

Animals

Male Sprague–Dawley rats (280–400 g body weight) were housed under a 12:12-h light:dark cycle, at controlled room temperature with food and water *ad libitum*. Experiments were conducted in accordance to the Guide for the Care and Use of Laboratory Animals of the National Research Council (USA, 1996). The 3 Rs principle of reduction, refinement and replacement were taken into account in the design of the animal experiment and applied where possible.

Treatments

We worked in two experimental sets as summarized in Figure 1.

Pretreatment with EFV to establish the model of chronic administration

Five doses of pF127-EFV micelles (drug dose of 20 mg/kg, group called EFV) or empty micelles (group called Vehicle) were administered to rats by gavage every 24 h over 5 days ($n = 4–6$). Between administrations, animals were housed with food and water *ad libitum*. In these groups of animals, pharmacokinetic assays were performed 4 h (group called EFV) or 28 h (group called Washout) after the last of five daily oral administrations.

No pre-treatment

Pharmacokinetic studies (see below) were conducted in naive animals without any previous treatment ($n = 4–6$).

Pharmacokinetic analysis

In all experimental groups, rats were anaesthetized with chloralose (50 mg/kg, ip.) and urethane (500 mg/kg, ip.) and cannulized in the femoral vein for intravenous administration of pF127-EFV micelles (dose of 20 mg/kg; control). Hippocampal and plasma samples were collected every 15 or 30 min, respectively, for 2 h. To evaluate the performance of F127:T904-EFV micelles that contain the ABCG2 inhibitor T904, micelles were administered 4 h after the last oral dose by the iv. route (group called Mixed micelles). In addition, single-dose experiments were performed to evaluate effects of the selective ABCG2 inhibitor gefitinib (20 mg/kg) or T904 (10%, the dose was identical to the one achieved in the administration of F127:T904-

EFV micelles considering the weight of each animal) alone. In these experiments, gefitinib and T904 were administered ip. 30 min before the iv. administration of pF127-EFV. In order to minimize the number of animals employed in the study, EFV concentrations in the CNS were monitored by a microdialysis technique [30] that employs a semipermeable micron-sized membrane inserted into the anterior hypothalamus by means of stereotaxy (A/P –5.2 mm, L/M 4.8 mm, V/D 7.5 mm, from bregma) [31]. Concentric microdialysis probes were home-made using fibers of cuprammonium rayon (2 mm long, o.d. 200 μ m and 10,000 molecular weight cut-off, Asahi Kasei Medical Co., Tokyo, Japan), stainless steel tubing (25 G) and silica tubing (o.d. of 145 μ m). The microdialysis probe was perfused with Ringer solution consisting in 147 mM NaCl, 2.4 mM CaCl_2 , 4.0 mM KCl, pH 7.3, at a rate of 2 μ l/min. An equilibration period of 2 h preceded drug administration in order to minimize BBB disruption [32]. The *in vitro* recovery of the microdialysis probe was determined after each experiment. A relative exposure index (AUCrel) was calculated by taking the ratio between AUC0–2 values in CNS and plasma, respectively.

Isolation of brain capillaries

Isolation of rat brain capillaries was performed as described by Miller *et al.* with modifications [33]. In brief, anaesthetized rats were decapitated, brains were taken immediately and kept at 4°C in Dulbecco's phosphate-buffered saline (DPBS, 2.7 mM KCl, 1.46 mM KH_2PO_4 , 136.9 mM NaCl and 8.1 mM Na_2HPO_4) supplemented with 5 mM D-glucose and 1 mM sodium pyruvate (pH 7.4), dissected and homogenized in the same volume of buffer. After addition of Ficoll (final concentration of 15%; Sigma-Aldrich), the homogenate was centrifuged at 5800 g for 20 min at 4°C, and the pellet was resuspended in DPBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich) and then passed over a glass-bead column. Capillaries adhering to the glass beads were collected by gentle agitation in DPBS with 1% BSA. Isolated brain capillary-enriched fractions containing tangled skeins of microvessels (as confirmed under light microscopy) were washed three-times with BSA-free DPBS buffer and stored at –70°C until use.

Western blot analysis

Brain capillaries were homogenized and lysed in mammalian tissue lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After 1 h on ice and occasional vortexing, samples were centrifuged at 10,000 g for 30 min. Protein samples were heated (95°C, 5 min)

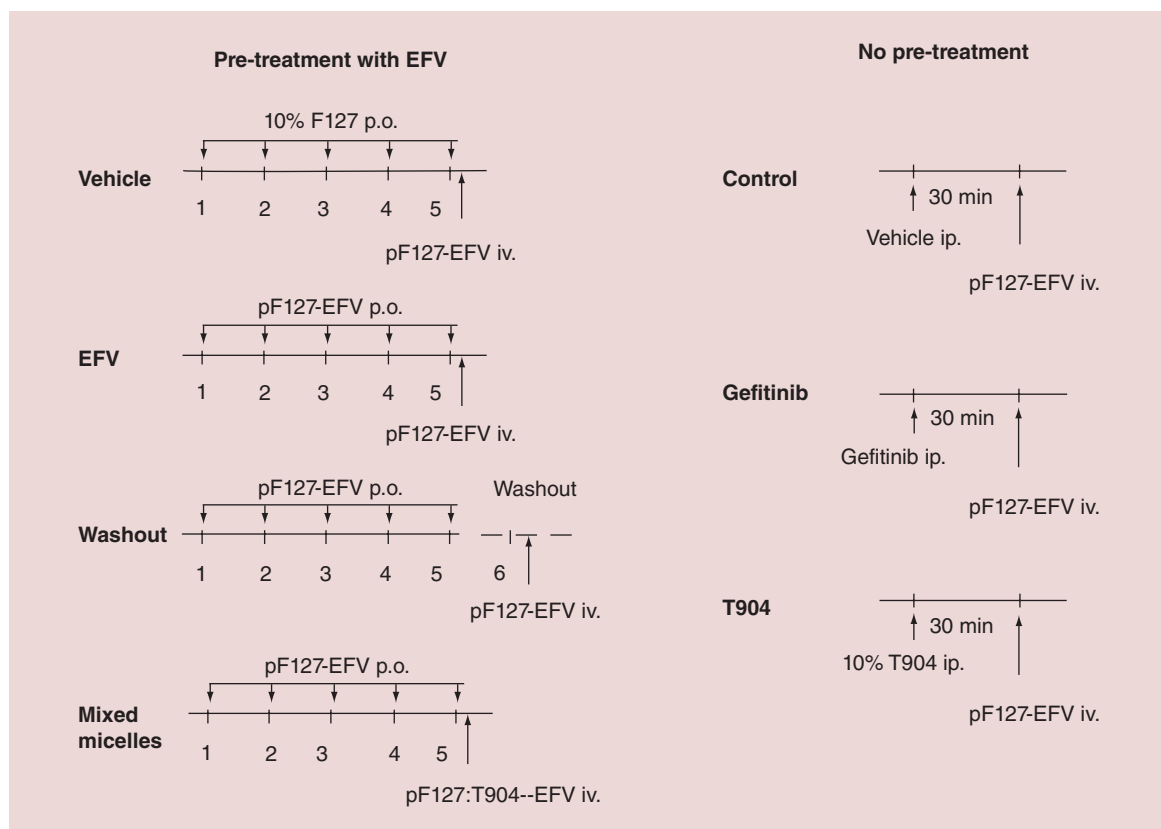


Figure 1. Scheme of dosing regimens and experimental groups.

EFV: Efavirenz; ip.: Intraperitoneally; iv.: Intravenously.

and 80 µg per line were loaded onto 8% SDS-polyacrylamide gels and subjected to electrophoresis. Then, gels were transferred to nitrocellulose membranes and blocked (room temperature, 1 h) with Tris buffered saline containing 0.3% Tween 20 and 5% nonfat dry milk. Blots were incubated over night at 4°C with rabbit polyclonal ABCG2 antibody (M-70, 1/400) or ABCB1 antibody (M-70, 1/800) (Santa Cruz Biotechnologies, CA, USA). A load control was performed with antiactin (A2066, 1/1000) from Sigma-Aldrich. The immune complexes were detected by incubation with the horseradish peroxidase-linked secondary antibody (1:2000) for 90 min. The bands were detected by autoradiography using enhanced chemiluminescence (Amersham ECL Biosciences, Amersham, UK) and quantified by densitometric analysis using ImageJ software (1.34S, US National Institutes of Health, MD, USA).

Quantification of EFV in biological samples

Samples were analyzed by high performance liquid chromatography (HPLC) using a Phenomenex Luna 5 µm, C18, 150 × 4.60 mm column (Phenomenex, Inc., CA, USA) with a UV detector (248 nm, UVIS 204, Linear Instruments, NV, USA) according to a technique described previously [25]. The mobile phase

was a mixture of distilled water:acetonitrile:triethylamine (60:40:0.2, pH 3) pumped at a flow rate of 1.4 ml/min. The analytical method for quantification was validated in the range of 20–5000 ng/ml.

Statistical analyses

Data are presented as the mean ± SEM ($n = 4-6$). Plasma versus hippocampal EFV profiles were analyzed by two-way analysis of variance followed by Bonferroni's *ad hoc* post-test. Residual concentrations after oral treatment were analyzed by Student *t*-test. AUC_{rel} and western blot assays were analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test.

Results

EFV pharmacokinetics in plasma & CNS after different chronic treatments

Rats were treated with pF127 or pF127-EFV by the oral route over 5 days according to a dosing regimen detailed in Figure 1. This scheme was based on previous reports showing that under these conditions, EFV leads to the overexpression of intestinal ABC transporters and thus, it was considered a chronic regimen [22].

Figure 2 shows the hippocampal concentrations of EFV 4 h after the last oral administration. Val-

ues were significantly lower than the obtained in the first point of the plasmatic ($7.723 \pm 1.065 \mu\text{g/ml}$) and hippocampal ($0.343 \pm 0.055 \mu\text{g/ml}$) profiles immediately after the iv. administration. Thus, the contribution of residual EFV was negligible. Then, animals received a single iv. dose of EFV and the concentrations followed up in both plasma and CNS. **Figure 3** presents hippocampal and plasmatic profiles of EFV in Vehicle (**Figure 3A**) and EFV (**Figure 3B**) groups, sampled at intervals of 15 and 30 min for 2 h, respectively. Significantly different EFV concentrations between plasma and hippocampus were observed in both groups (**Figure 3A & B**). The pharmacokinetic analysis indicated that the chronic administration of pF127-EFV led to a sharp decrease of the relative bioavailability of the drug in the CNS with respect to the control treated with pF127. In addition, the washout period of 24 h recovered the CNS bioavailability (**Figure 3C**). In other words, values of area under the curve (AUC) indicated that EFV plasma concentrations were more than twice higher in EFV-treated animals (EFV group) than in vehicle-treated counterparts (Vehicle group). On the other hand, the accumulation of EFV in the CNS, expressed as AUC_{0-2} , was similar in both groups (**Table 1**). To compare the relative brain exposure for different experimental groups, the AUC_{rel} was calculated. As shown in **Figure 3D**, the AUC_{rel} underwent a sharp decrease in animals chronically treated with EFV compared with vehicles; values being 0.283 ± 0.070 and 0.072 ± 0.010 for EFV and Vehicle groups, respectively. After a washout period of 28 h (**Figure 1**), EFV was not detected neither in CNS nor plasma by HPLC-UV. The iv. administration of single micelles (pF127-EFV) resulted in higher (though nonstatistically significant) AUC_{0-2} CNS values in the washout group ($0.700 \pm 0.088 \text{ g}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$) than those measured 4 h after the last oral administration ($0.567 \pm 0.087 \text{ g}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$) (**Table 1**). Conversely, AUC_{0-2} in plasma was $4.151 \pm 0.371 \text{ g}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$, this value being significantly lower than the one obtained before washout ($6.859 \pm 0.494 \text{ g}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$) (**Table 1**). These findings were confirmed by the sharp recovery of the AUC_{rel} in the Washout group to values that were similar to those found in vehicles (0.300 ± 0.063 and 0.283 ± 0.070 for washout and vehicle, respectively) (**Figure 3D**).

Expression of ABCB1 & ABCG2 transporters in the BBB

Since the distribution of drugs into the CNS is governed by the presence of ABC transporters, mainly ABCB1 and ABCG2 in brain capillary endothelial cells, their expression levels were analyzed by Western blotting (**Figure 4**). The expression of ABCG2 in BBB

increased significantly after chronic treatment with EFV, this increase being transient and reverting after the washout period (**Figure 4B**). By contrast, ABCB1 expression levels in BBB remained unchanged under every experimental condition (**Figure 4C**).

Passage of EFV into the CNS in presence of ABCG2 inhibitors

Aiming to study the role of ABCG2 in the passage of EFV into the CNS, single-dose assays were conducted in a group of naive animals pretreated with the highly active and selective pharmacological ABCG2 inhibitor gefitinib (20 mg/kg , ip.) (**Figure 1**). **Figure 5B** shows that gefitinib increased the bioavailability of EFV in the CNS after iv. administration with respect to controls (**Figure 5A**) without apparent modifications of the plasma concentrations. Moreover, the AUC_{rel} was increased by approximately fourfold, from 0.225 ± 0.017 in the control to 0.834 ± 0.102 in gefitinib-treated animals (**Figure 5D**). Outstandingly, plasma and CNS concentrations in gefitinib-administered rats were almost superimposable.

The inhibition of the functional activity of ABCB1 (P-gp) and ABCG2 (BCRP) by T904 was demonstrated in different cell lines [23,24]. Thus, we also investigated the effect of the ip. preadministration of 10% T904 polymeric micelles (a copolymer dose that was equivalent to the one in the mixed polymeric micelles and adjusted to the animal weight) in the pharmacokinetics profile of EFV after a single dose of the drug. T904 boosted the passage of EFV (administered iv. in pF127-EFV micelles) into the CNS (**Figure 5C**) and resulted in an AUC_{rel} value of 0.547 ± 0.010 (**Figure 5D**).

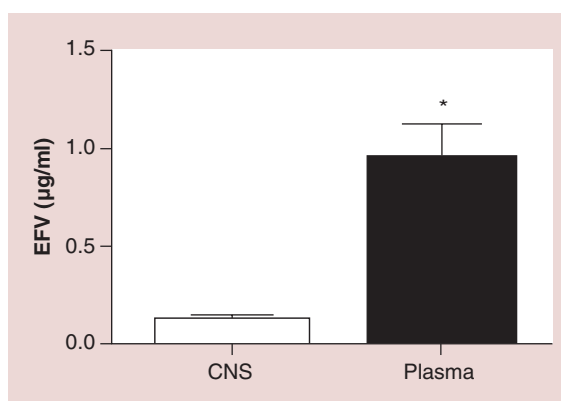


Figure 2. Residual concentrations of efavirenz.

Quantification of plasma as well as hippocampal levels of EFV 4 h after last oral administration of pF127-EFV in EFV group. Samples were taken from the hippocampus (CNS) and from the tail vein (plasma). Data are presented as mean \pm SEM of the μg EFV/ml ($n = 6$). * $p < 0.05$ between EFV concentrations in CNS and plasma.

EFV: Efavirenz.

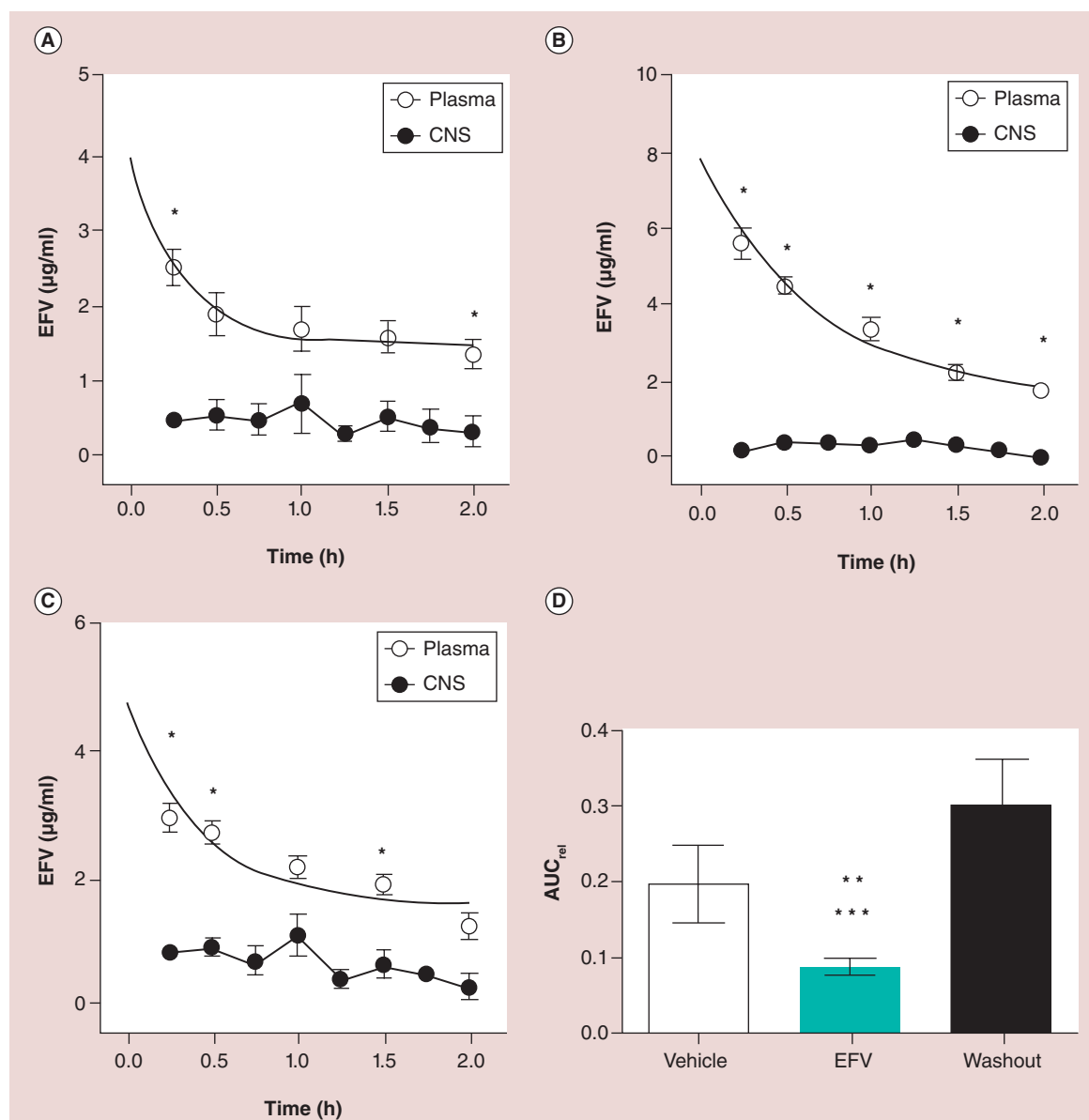


Figure 3. Efavirenz pharmacokinetics in plasma and CNS. (A–C) Effect of repeated oral administration of EFV on its pharmacokinetic in plasma and CNS. After an iv. administration of pF127-EFV, plasma (white circles) and hippocampal (black circles) samples were collected every 15 or 30 min, respectively, for 2 h. The experiments were performed in vehicle (empty pF127 micelles, 5 days, p.o.) (A) and EFV-treated animals (pF127-EFV, 5 days, p.o.) 4 h (B) or 28 h (C) after last oral administration. Data are presented as mean \pm SEM of the μg EFV/ml, * $p < 0.05$ between plasma and CNS in indicated time points (A–C). In (D): Distribution of EFV in the CNS expressed as AUC_{rel} ($\text{AUC}_{0-2\text{CNS}} / \text{AUC}_{0-2\text{Plasma}}$ ratio) for vehicle (white bar) as well as EFV-treated animals 4 h (dotted bar, 'EFV') or 28 h (hatched bar, 'washout') after last oral administration. Data are presented as mean \pm SEM of the AUC_{rel} ($n = 4-6$). ** $p < 0.001$ between EFV and washout; *** $p < 0.001$ between vehicle and EFV. AUC: Area under the curve; EFV: Efavirenz.

that was significantly greater than the control and nonstatistically different from the gefitinib group.

Passage of EFV into the CNS in polymeric micelles containing the ABCG2 inhibitor T904

Given the results obtained with T904 pretreatment and the fact that F127:T904-EFV micelles boosted the intestinal and the intranasal absorption of the drug

with respect to micelles without T904 [25–26,29], we assessed the effect of these mixed micelles of identical composition [26,29] on the CNS bioavailability of EFV after iv. administration (Figure 1). The AUC_{rel} with F127:T904-EFV micelles was significantly greater than in pF127-EFV, values being 0.471 ± 0.103 and 0.078 ± 0.009 , respectively ($p < 0.01$) and representing a sixfold increase (Figure 6).

Discussion

EFV is a first-line antiretroviral in the pharmacotherapy of adult and pediatric HIV patients. Current clinical experience has revealed important differences in patient response to the treatment, which largely can be attributed to high inter- and intraindividual variability. This supports the TDM to optimize viral load suppression and to minimize CNS toxicity [15]. However, this practice is complex, especially in constrained-setting countries. Moreover, low bioavailability in the CNS contributes to the development of neurocognitive disorders.

The present work investigated the involvement of ABCG2 in the efflux of EFV in the BBB and a clinically translatable nanotechnology strategy to overcome this pathway based on the administration of polymeric micelles that play two roles, nanoencapsulation of the poorly water-soluble EFV and inhibition of ABCG2 in the BBB.

To achieve this, a preclinical model of overexpressed ABCG2 in the BBB was established by the daily oral administration of EFV-loaded polymeric micelles over 5 days. This dosing regimen was based on previous data showing that under these conditions, EFV leads to an overexpression of ABCG2 in the intestinal epithelium [22]. The use of micelles as nanocarrier was fundamental to ensure sufficiently high EFV concentrations in the CNS that would be quantifiable employing HPLC.

First, quantitative microdialysis in hippocampus allowed us to demonstrate that concentrations of EFV in CNS were lower than those found in plasma in both vehicle- and EFV-treated animals. Since EFV is a very lipophilic molecule that can readily enter the CNS, our results supported the existence of an active transport mechanism against the concentration gradient that

restricts its accumulation in the CNS and leads to a high plasma-to-CNS ratio. With respect to the significant increase in EFV plasma levels after repeated oral administration of the drug, this could be because it undergoes metabolism primarily by cytochrome P450 (CYP) 2B6, with some involvement of CYP3A and it has a long plasma half-life [34]. Balani *et al.* demonstrated that at dose higher than 15 mg/kg EFV in rats shows nonlinear pharmacokinetics accompanied by a reduced drug disposition implying saturation of metabolism processes that leads to disproportionate increases in the AUC [35]. Furthermore, the significant decrease in EFV brain distribution clearly shows a change in the proportion between plasma and central drug levels after chronic treatment; this was confirmed by the fact that the AUC_{rel} was markedly lower in animals treated with pF127-EFV over 5 days than controls receiving the vehicle. A washout period of 28 h after which EFV was undetectable in both plasma and CNS restored the CNS/plasma concentration ratio found in the pF127-treated animals. These results are in agreement with epidemiologic studies showing that the majority of neuropsychiatric adverse effects that can lead to poor adherence, treatment interruptions or change of antiretroviral therapy regimens have been associated with the high variability in the penetration of EFV into the CNS [36].

The penetration of xenobiotic compounds into the brain is restricted by the BBB formed by brain capillary endothelial cells. The presence of active carrier-mediated transport of substrates from the brain to the blood is a major feature of the barrier properties of the BBB. A number of efflux transport systems expressed and polarized on the luminal or the ab-luminal surface of the BBB (e.g., ABCs) play a critical role in preventing the passage of drugs and neurotoxic substances into

Table 1. AUC_{0-2} for efavirenz in plasma and CNS after the different treatments.

Treatments	AUC_{0-2} plasma (g.ml ⁻¹ .h ⁻¹)	AUC_{0-2} CNS (g.ml ⁻¹ .h ⁻¹)
Chronic treatment		
Vehicle	3.514 ± 0.468	0.623 ± 0.116
EFV	6.859 ± 0.494****	0.567 ± 0.087
Washout	4.151 ± 0.371***	0.700 ± 0.088
Mixed micelles	5.651 ± 1.401	2.361 ± 0.571**
Single-dose treatments (no pre-treatment)		
Control	2.464 ± 0.643	0.650 ± 0.187
Gefitinib	3.397 ± 0.993	3.011 ± 1.042*
T904 polymeric micelles.	2.168 ± 0.376	1.710 ± 0.327
*p < 0.05 compared with control. **p < 0.01 compared with EFV. ***p < 0.001 compared with EFV. ****p < 0.0001 compared with vehicle. AUC: Area under the curve; EFV: Efavirenz.		

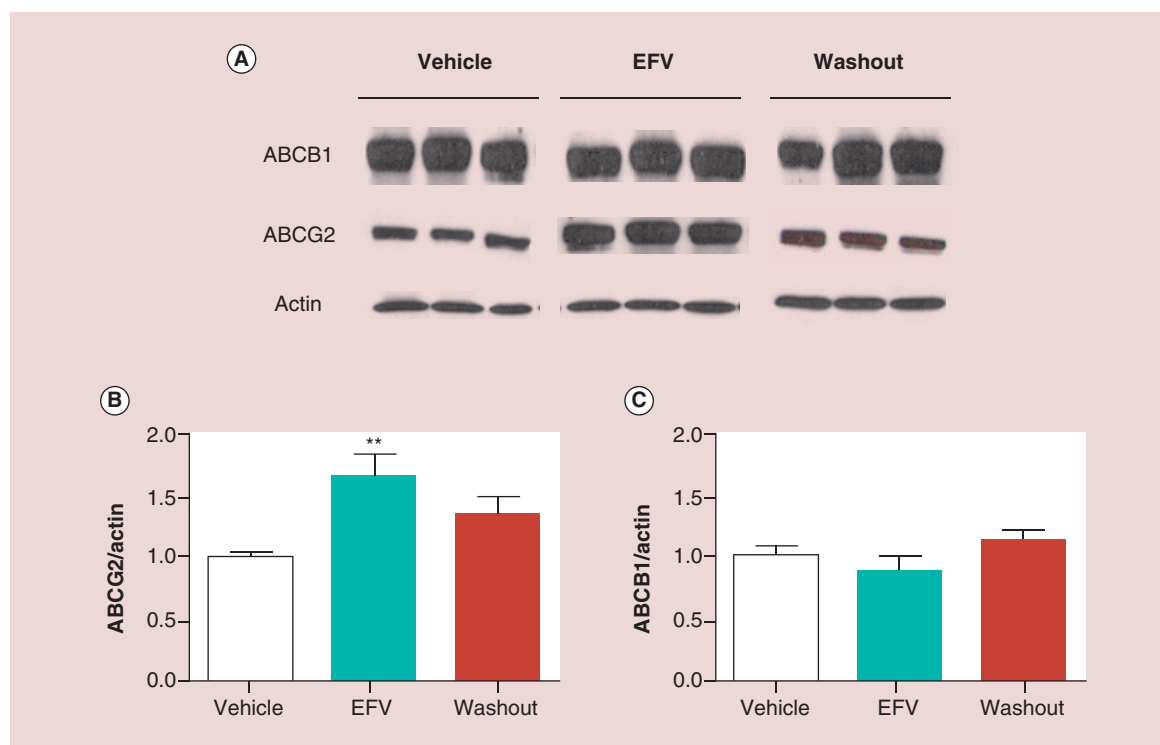


Figure 4. Effect of repeated oral administration of efavirenz on the expression levels of ABCG2 and ABCB1 in the blood–brain barrier. (A) Representative immunoblot for ABCB1, ABCG2 and actin performed in endothelial cells of brain capillaries obtained 6 (EFV) or 30 h (washout) after last oral administration of pF127-EFV or the vehicle (pF127). Equal amounts of total protein (80 μ g) were loaded in all lanes. Relative densitometric units were calculated as ratio between optical density of ABCB1 and actin (B) or ABCG2 and actin (C). Data are presented as media \pm SEM (n = 4–6).

**p < 0.01 between EFV and vehicle.

EFV: Efavirenz.

the brain and their presence challenges the attainment of therapeutic concentrations. ABCG2 and ABCB1 are two ABCs highly expressed in the BBB [37].

Chronic EFV administration led to a sharp increase of the ABCG2 expression that in turn reduced the CNS bioavailability of the drug (present results). At the same time, it is worth stressing that even though under the experimental conditions used in this work, EFV did not alter the expression of ABCB1, the participation of this transporter in its efflux could not be completely ruled out. In fact, previous *in vitro* studies demonstrated that the chronic treatment with EFV could indirectly induced ABCB1 by activation of ligand-activated nuclear receptors known to regulate the expression of this efflux pump [12]. On the other hand, present results are in accordance with those obtained in the gut, where the permeability of EFV was exclusively mediated by ABCG2 [22].

Confirmation that ABCG2 restricted the distribution of EFV in the brain was given by the fact that the administration of the highly selective inhibitor gefitinib produced a significant increase in hippocampal EFV concentrations, to the point that equaled those

obtained in plasma, and raised AUC_{rel} values to 0.834, which means that EFV almost freely entered into the CNS from plasma.

At the same time, even when a cornucopia of pharmacologically active compounds were identified as ABC inhibitors over the last decades, clinical results mainly oriented to overcome multidrug resistance in cancer have been inconclusive mostly owing to high toxicity and/or low efficiency. Gefitinib is a selective inhibitor of epidermal growth factor that plays a pivotal role in the control of cell growth, apoptosis and angiogenesis and thus it is far from harmlessness [38]; the main adverse effects of gefitinib are diarrhea, dry skin, rash, nausea and vomiting that need appropriate intervention to revert. Therefore, nowadays the rational search for ‘innocuous’ inhibitors of ABC transporters effective and safe is focused on natural herbal extracts, pharmaceutical excipients and formulations based on nanotechnology [17,39].

According to these assumptions and given that inhibition of ABCB1 and ABCG2 by T904 was previously shown in various cell lines [23,24], we also tested the effect of the preadministration of 10% T904 (ip.) on

the pharmacokinetics of EFV. Interestingly, the passage of EFV into the CNS was significantly increased, providing solid evidence that this copolymer is an effective ABCG2 inhibitor not only *in vitro* but also *in vivo*. In this context, a wake-up call is mandatory

as many poly(ethylene oxide)-*b*-poly(propylene oxide) copolymers are classified as ‘Generally Recognized As Safe’ by the US FDA [17]. Their lack of inertness and well as that of many other pharmaceutical excipients should be thus comprehensively revisited. Even more

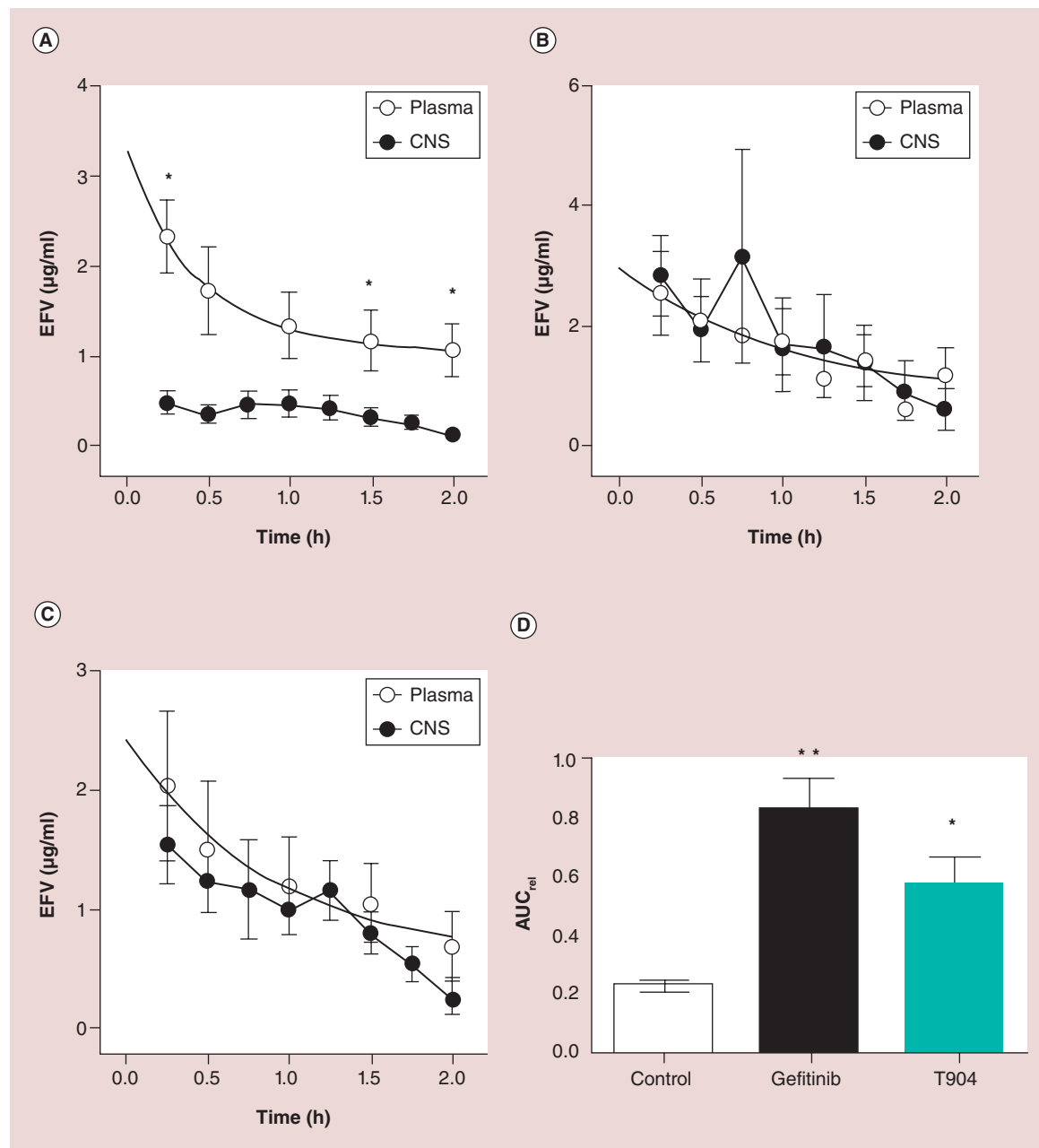


Figure 5. Effect of the ABCG2 inhibitor gefitinib and T904 preadministration on the pharmacokinetics of efavirenz in plasma and CNS. After an iv. administration of pF127-EFV, plasma (white circles) and hippocampal (black circles) samples were collected every 15 or 30 min, respectively, for 2 h. Gefitinib (20 mg/kg ip. [B]) or T904 (10% ip. [C]) or vehicle (A) were administered 30 min before starting experiments. Data are presented as mean \pm SEM of the μ g EFV/ml, * $p < 0.05$ between plasma and CNS for in indicated time points in control group (A). (D) Distribution of EFV in CNS expressed as AUC_{rel} (AUC_{0–2CNS}/AUC_{0–2Plasma} ratio) for vehicle (control: white bar), gefitinib (dotted bar) or T904 (hatched bar). Data are presented as mean \pm SEM of the μ g of EFV/ml (A–C) and of AUC_{rel} (D) ($n = 4–6$).

* $p < 0.05$.

** $p < 0.001$ between gefitinib or T904 and control.

AUC: Area under the curve; EFV: Efavirenz.

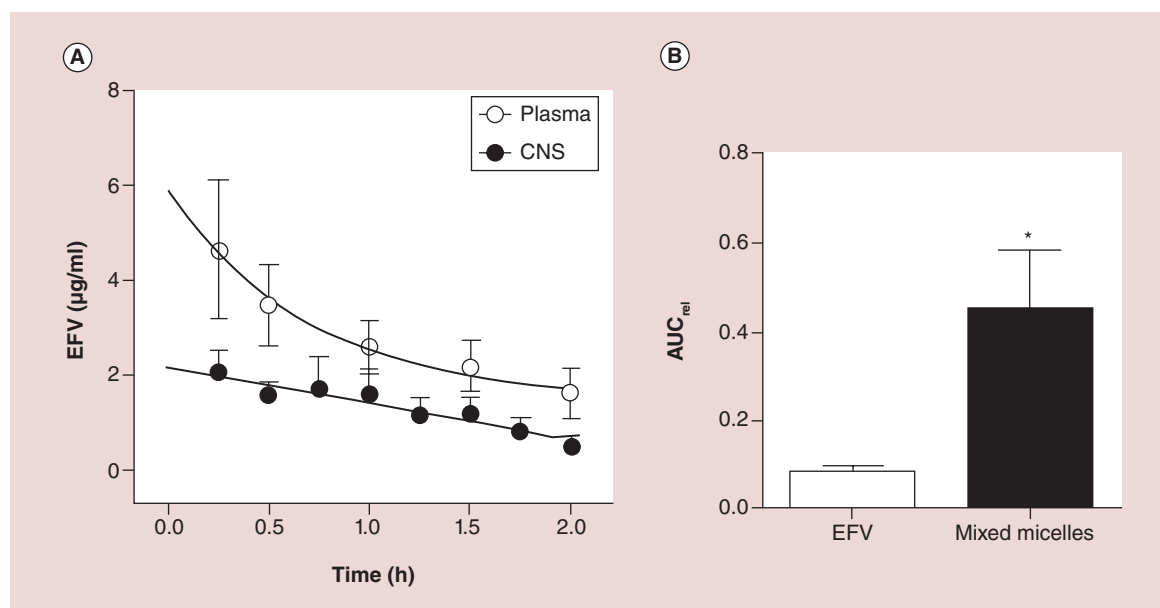


Figure 6. Effect of F127:T904-efavirenz micelles on the pharmacokinetics of efavirenz after chronic treatment.

(A) After iv. administration of F127:T904-EFV (mixed micelles), plasma (white circles) and hippocampal (black circles) samples were collected every 15 or 30 min, respectively, for 2 h. The experiments were performed in pF127-EFV-treated animals 4 h after last oral administration. (B) Distribution of EFV in CNS expressed as AUC_{rel} ($AUC_{0-2CNS}/AUC_{0-2Plasma}$ ratio) for EFV (repeated from Figure 3 for the sake of comparison) and mixed micelles. Data are presented as mean \pm SEM of the μ g of EFV/ml and of AUC_{rel} ($n = 5$).

* $p < 0.05$ between AUC_{rel} of EFV and mixed micelles.

AUC: Area under the curve; EFV: Efavirenz.

relevant are the results with mixed micelles containing T904 that confirmed previous studies indirectly suggesting that this specific copolymer inhibits the activity of ABCs *in vivo* even when incorporated into polymeric micelles [26,29]. This behavior probably stemmed from the fact that these nanocarriers are self-assembled and always in dynamic equilibrium with a low concentration of free copolymer molecules (unimers) that would be responsible for the inhibition.

Conclusion

In this work, we have investigated the ability of the copolymeric amphiphile T904 to inhibit ABCG2 in the BBB and by doing so to increase the bioavailability of the antiretroviral EFV in the CNS. Overall, our findings highlight the relevance of ABCG2 in the biodistribution of EFV in the CNS and provides a simple nanotechnology-based strategy to overcome its overexpression that leads to subtherapeutic concentrations and uncontrolled neuropsychiatric complications due to high viral concentrations. This simple and translatable strategy could be further exploited not only in the improvement of the HIV therapy but also of other diseases of CNS.

Future perspective

Drug transporters have become increasingly important in the research of novel drugs and pharmaceutical formulations due to the major role they play in

absorption, distribution and excretion of endogenous and exogenous compounds, inasmuch as transporter-mediated drug–drug interactions (DDI) are associated with potential toxicological and pharmacological consequences [40]. Recent guidance documents released by the US FDA and the EMA emphasize the importance of evaluating the potential of new drug candidates for transporter-mediated DDI with a determination of victim (substrate) and perpetrator (inhibitor) potential [40–42]. In consequence, a proof-of-concept clinical study is being planned by a collaborative consortium to evaluate the proposed BCRP DDI study design [43]. However, these recommendations are very recent and there are not available drugs that have been designed in accordance with the assumptions set out above. Thus, conceiving innovative strategies to optimize the treatment of disease with drugs available on the market is of great value, especially when a direct translation into the clinical phase is envisioned. In this sense, the extensive development that has had clinical pharmacology, dealing with the effectiveness and safety of drugs in humans [44] has enabled the improvement of pharmacotherapy against HIV in children and pregnant women using the TDM as a fundamental tool [45–49]. However, it is known that drug levels in plasma often not reliably correlate with the accumulation thereof in tissues separated by barriers such as the BBB. It is for this reason that in the

last decade a large number of investigations that focus on nanotechnology have been developed. Regenerative, protective, immune modulatory, antimicrobial and anti-inflammatory products or imaging agents are readily encapsulated in or conjugated to nanoparticles and as such facilitate the delivery of drug payloads to specific action sites across the BBB [49] in order to optimize pharmacotherapy.

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Ethical conduct

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

EFV pharmacokinetics in plasma and CNS after different chronic treatments

- Chronic oral administration of efavirenz (EFV) led to a sharp decrease of the CNS bioavailability of the drug with respect to plasma.

Expression of ABCB1 and ABCG2 transporters in the blood–brain barrier

- The expression of ABCG2 in blood–brain barrier increased significantly after chronic treatment with EFV, while ABCB1 expression levels in BBB remained unchanged under every experimental condition.

Passage of EFV into the CNS in presence of ABCG2 inhibitors

- Administration of the highly selective inhibitor gefitinib and the ABCG2 polymeric inhibitor T904 (ip.) significantly increased hippocampal EFV concentrations.

Passage of EFV into the CNS in polymeric micelles containing the ABCG2 inhibitor T904

- T904-containing polymeric micelles also boosted the bioavailability of EFV in the CNS, strongly supporting that T904-containing micelles play two roles, nanocarrier of the poorly water-soluble EFV and inhibitor of ABCG2 in the blood–brain barrier *in vivo*.

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